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Research article

Induction of defense-related enzymes in patchouli inoculated with virulent *Ralstonia solanacearum*

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ABSTRACT

Background: Defense-related anti-oxidative response is a vital defense mechanism of plants against pathogen invasion. *Ralstonia solanacearum* is an important phytopathogen. Bacterial wilt caused by *R. solanacearum* is the most destructive disease and causes severe losses in patchouli, an important aromatic and medicinal plant in Southeast Asia. The present study evaluated the defense response of patchouli inoculated with virulent *R. solanacearum*.

Results: Results showed that the basic enzymatic activities differed not only between the leaves and stems but also between the upper and lower parts of the same organ of patchouli. POD, SOD, PPO, and PAL enzymatic activities were significantly elevated in leaves and stems from patchouli inoculated with *R. solanacearum* compared to those in control. The variation magnitude and rate of POD, PPO, and PAL activities were more obvious than those of SOD in patchouli inoculated with *R. solanacearum*. PAGE isoenzymatic analysis showed that there were one new POD band and two new SOD bands elicited, and at least two isoformic POD bands and two SOD bands were observably intensified compared to the corresponding control.

Conclusion: Our results suggest that not only defense-related enzymatic activities were elevated but also the new isoenzymatic isoforms were induced in patchouli inoculated with *R. solanacearum*.

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1. Introduction

Pogostemon cablin (patchouli) is an important aromatic plant belonging to the family Lamiaceae, native to southeast Asia, and is now extensively cultivated in many tropical and subtropical countries, especially China, Indonesia, India, Brazil, Vietnam, Malaysia, Mauritius, Philippines, and Thailand. Patchouli oil, which is extracted from its leaves, is widely used in the modern perfume, cosmetic, and food industries. Modern research has repeatedly demonstrated the various pharmacological activities of this oil including anti-inflammatory, antiseptic, antibacterial, antifungal, antidepressant, and insect repellent properties [1]. In China, patchouli is also a famous herbal remedy that has long been used to treat common cold, nausea, pain, headaches, infections, and digestive problems.

Today, growing interest in its fragrance and medicinal applications has led to patchouli's widespread cultivation in southern China. However, patchouli is very sensitive to bacterial wilt, a serious disease caused by *Ralstonia solanacearum*. The genetic variability within patchouli is relatively limited; therefore, breeding potential for resistance to biotic stresses is also limited. Patchouli is propagated by stem cuttings, which further limits its available genetic pool. Furthermore, stem cuttings can harbor the pathogens, thus allowing the disease to be perpetuated through vegetative propagation. Despite various attempts in the past decades, effective prevention and control of patchouli bacterial wilt disease to date has hardly been obtained. Therefore, it is necessary to find a potential new strategy for protecting patchouli against attacks by *R. solanacearum*.

Defense-related enzymes constitute an important protective system for plants against pathogen invasion. In recent years, intensive efforts have been devoted to the elucidation of defensive responses to pathogen invasion [2,3,4,5,6,7,8]. Current research has determined superoxide dismutase (SOD), peroxidase (POD), polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL) as vital defense-related enzymes of plants, and therefore, these enzymes are extensively studied in the research of plant defense against phytopathogens [9,10,11,12]. SOD, POD, and PPO are representative antioxidant enzymes and are important components of defense against membrane lipid peroxidation and oxidative stress during pathogen invasion [13]. SOD is

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the first enzyme involved in the antioxidant defense. The ability of plants to overcome oxidative stress partly relies on the induction of SOD activity and subsequently on the upregulation of other downstream antioxidant enzymes in the defense [14]. POD is an oxidoreductive enzyme that participates in cell wall polysaccharide processes such as oxidation of phenols, suberization, and lignification of host plant cells during defense reaction against pathogenic agents [15]. PPO is involved in the oxidation of polyphenols into quinones and lignification of plant cells during microbial invasion [16]. PAL is the primary enzyme in phenylpropanoid metabolism and plays an important role in the synthesis of several defense-related secondary compounds such as phenols and lignin [17].

R. solanacearum is an important phytopathogen and a causal agent of devastating plant wilt disease. Bacterial wilt caused by *R. solanacearum* is a serious soil-borne disease of many economically important crops, such as tomato, potato, tobacco, eggplant, and banana. Defense responses to *R. solanacearum* involving antioxidant enzymes were reported in some crops, especially plants of the *Solanaceae* family, such as tomato, potato, and eggplant. However, the study of defense response to *R. solanacearum* on its new host, especially medicinal plants such as patchouli has rarely been reported to date. *R. solanacearum* is a “species complex” with high heterogeneity on various hosts [18,19]. Currently, increasingly more numbers of *R. solanacearum* strains are being isolated from different plants. In addition, some new hosts of *R. solanacearum*, such as patchouli, were reported [20]. However, evidence for the induction of defense enzymes that protect plants from infection by the bacterium in new hosts is still preliminary and inconsistent. In the present work, attempt has been made to investigate the defense response of patchouli inoculated with *R. solanacearum*, which was isolated from a naturally wilted patchouli plant grown in Guangdong, China.

2. Materials and methods

2.1. Plant materials and culture

Leaves procured from elite patchouli mother plants (grown in Guangzhou, China, E 112°57′13″, N 222°6′35″) were washed 3 to 4 times in tap water and surface sterilized for 1 min in 75% ethanol, followed by immersion in 0.1% (*w*/*v*) $HgCl_2$ solution for 5 min, and finally rinsed thoroughly with sterile distilled water. *In vitro* regeneration of plantlets was achieved through a series of culturing steps under the following specified conditions: (1) for shoot initiation and multiplication, surface-disinfected explants were inoculated on full strength solid MT basal medium supplemented with 0.2 mg/L 6-benzyladenine and 3% (*w*/*v*) sucrose for 4 weeks.

Rapid and prolific shoot regeneration through direct somatic embryogenesis was initiated from leaf explants of patchouli after initial culturing; (2) for root development and true-to-type plantlets, profuse rooting from regenerated shoots was induced by transferring uniform proliferated shoots to MT medium devoid of phytohormones after 2 months. Established cultures were subcultured in the same medium at 4-week intervals. All the cultures were maintained at 25 ± 2°C under a 12-h photoperiod with a light intensity of 3000 lx provided by cool white fluorescent tubes. Seven to eight-week-old plants exhibiting 12–14 leaves were used for the experiments. Leaves were numbered according to their time of appearance. Leaves located at the base of the stem were numbered from 1 to 6, designated as lower parts, and those above were numbered from 7 to 12. Patchouli plantlets were sampled thrice for each treatment for the examination of enzymes. All plantlets were initially of identical length and similar size.

2.2. R. solanacearum and culture

In our previous investigation, a strain of *R. solanacearum* was isolated from the vascular bundles of patchouli affected by bacterial wilt in Guangdong Province, China [20]. The strain was routinely grown or maintained at 28°C on NA plate (beef extract 3 g/L, yeast extract 1 g/L, bacterial peptone 10 g/L, sucrose 10 g/L, pH 7.2). Stock cultures of *R. solanacearum* were preserved at −20°C in NA liquid medium containing 7% dimethyl sulfoxide prior to use. The strain was routinely checked for any contamination during this investigation.

2.3. Inoculation of patchouli

A single colony of *R. solanacearum* was inoculated in nutrient broth and incubated in a shaking incubator at 200 rpm overnight and 28°C for 12 h (logarithmic phase). The density of the bacterial suspension for inoculation was prepared and adjusted to 1.0×10^8 CFU/mL. The suspensions were immediately used for the inoculation of patchouli.

Roots of patchouli plantlets were inoculated to evaluate its association with the phytopathogenic bacteria *R. solanacearum*. Bacterial suspensions of 1 mL volume were inoculated into the roots of patchouli by the root wounding method. Plants infiltrated with aliquots of sterile distilled water served as controls. At the end of each treatment, plantlets inoculated with *R. solanacearum* were studied for the determination of defense enzymes of patchouli in the following days.

2.4. Enzyme extraction and assay

Each patchouli plantlet was separated into two parts, the upper half (top, leaves numbered from 7 to 12) and the lower part (bottom, leaves numbered from 1 to 6). Each part was further segregated into two tissues: leaf and stem. Leaves and stems (upper/lower, 0.2 g) were well-homogenized in a homogenizer in 2 mL ice-cold 50 mM sodium phosphate buffer (pH 6.0) for POD, PPO, and SOD assay and in 50 mM borate buffer (pH 8.8, containing 5 mM β-mercaptoethanol) for PAL assessment, individually. The homogenate was centrifuged at 12,000 × g for 10 min, and then the resultant supernatant was used for subsequent enzymatic activity assays. All operations were performed at 0°C to 4°C.

2.5. Peroxidase assay

POD (E.C. 1.11.1.7) activity was quantified by taking 0.1 mL homogenate in 3 mL of 0.1 M sodium phosphate buffer, pH 6.0 (containing 19 μL of 30% hydrogen peroxide (H_2O_2) as the oxidant and 38 μL guaiacol as the hydrogen donor per 50 mL buffer). The oxidation of guaiacol was measured by spectrophotometry at 470 nm and <25°C. The enzymatic activity (Units g^{-1} FW min^{-1}) was determined by measuring the maximum slope of the reaction line.

2.6. Superoxide dismutase assay

SOD (EC 1.15.1.1) activity was analyzed according to the method of Gay and Tuzun et al. [21], with the following modifications. The reaction mixture (3.0 mL) contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM nitroblue tetrazolium (NBT), 10 μM ethylenediaminetetra acetic acid disodium (EDTA-Na_2), 2.0 μM riboflavin (added last), and 0.1 mL homogenate in a final volume of 3 mL. The mixtures were illuminated by a fluorescent lamp (3000 lx) for 15 min, and the absorbance was measured immediately at 560 nm. Identical solutions held in the dark served as blanks. The reaction mixture without adding specific enzymes developed maximum color due to the highest reduction of NBT. One unit of SOD was defined as the amount of enzyme that caused a 50% decrease in the

SOD-inhibitable NBT reduction. The specific activity was expressed as units per gram fresh weight (FW) per min (units g^{-1} FW min^{-1}).

2.7. Polyphenol oxidase assay

PPO (EC 1.10.3.1) activity was assayed spectrophotometrically according to the method of Jiang et al., with some modifications [22]. The reaction mixture consisted of 0.1 mL homogenate and 3.0 mL substrate solution containing 0.1 M catechol as the substrate and 0.1 M sodium phosphate buffer (pH 7.40). The reference cuvette contained only the substrate. The rate of oxidation of catechol was monitored at 410 nm at 25°C for 1 min. PPO activity is presented as the change in unit optical density (OD, at 410 nm) g^{-1} FW min^{-1}.

2.8. Phenylalanine ammonia-lyase assay

PAL (EC 4.3.1.5) activity was assayed as follows: 1.0 mL homogenate was incubated with 1.0 mL of 50 mM borate buffer (pH 8.8) and 1.0 mL of 20 mM L-phenylalanine for 60 min at 37°C. The reaction was terminated by the addition of 0.1 mL of 6.0 M HCl. PAL activity was determined on the basis of the production of trans-cinnamate, measured by the absorbance change at 290 nm. The blank was the crude enzyme preparation mixed with L-phenylalanine with zero time incubation. One unit enzyme activity represents the amount of enzyme that produces 1.0 μmol of cinnamic acid per hour.

POD, SOD, PPO, and PAL of patchouli showed an absorption maximum at 470, 560, 410, and 290 nm, respectively, according to their ultraviolet–visible absorption spectra. In addition, the four enzyme activity assays were performed at nine different pH values (5.5, 6.0, 6.5, 7.0, 7.4, 7.8, 8.4, 8.8, and 9.0) to select the best sensitivity for their individual activities. Accordingly, pH 6.0, 7.8, 7.4, and 8.8 gave better resolution for the assay of POD, SOD, PPO, and PAL, respectively. Before the enzyme assay, the reaction systems were incubated in a water bath-thermostat that maintained their temperatures at 25°C.

2.9. Native PAGE and isoform staining for POD and SOD

Isoenzymatic activity was evaluated by discontinuous polyacrylamide electrophoresis (PAGE) under non-denaturing conditions. For the preparation of crude enzyme, 1.0 g FW of patchouli was homogenized in a homogenizer in 2.0 mL ice-cold 50 mM sodium phosphate buffer (pH 6.0, containing 0.1 mM EDTA). Enzyme extracts of patchouli were subjected to non-denaturing PAGE. Stacking gel concentration was 3% and the separation gel concentration was 10%. The electrode buffer was Tris-glycine (pH = 8.3). Preliminary studies indicated that this mixture of components resulted in the least difficulty in casting the gels and best separation of isozymes. Subsequently, 50 μL crude extracts were mixed with glycerol and bromophenol blue, applied to each sample well with a syringe, and allowed to diffuse into the gel for 5 min. Electrophoretic separation was performed at a constant voltage of 150 V in the stacking phase for 2 h and 200 V and in the separation phase for 3.5 h at 4°C. After completion of electrophoresis, the gels were stained immediately to determine the activities of POD and SOD.

Active staining of POD isozymes was achieved by incubating the gels in sodium acetate buffer (pH 4.5) containing 3.2 mM benzidine (dissolved in glacial acetic acid), 9.2 mM NH_4Cl, and 14.7 mM EDTA, which was found to be more sensitive and reliable than guaiacol for visualizing PODs in gels. The reaction was initiated by adding 5.0 mL of 3.0 mM H_2O_2, and the reaction was allowed to continue until bands were revealed thoroughly.

Activity staining of SOD isozymes was fulfilled as follows: gels were incubated in a solution comprising 2.5 mM NBT for 20 min, followed by incubation in 50 mM sodium phosphate buffer (pH 7.8) containing 28 μM riboflavin and 28 μM TEMED for 20 min in the dark. Then the gel was transferred to 50 mM sodium phosphate buffer (pH 7.8) for 20 min at room temperature. Visualization of isozymes was achieved by exposing the gels to light of 4000 lx, provided by cool white fluorescent tubes until SOD isoenzymes bands could be clearly observed on a dark blue background.

2.10. Statistical analysis

The experiment was set up in a completely randomized design. Data were presented as means ± standard deviations (S.D.). Statistical differences between the tested groups were analyzed by analysis of variance (ANOVA) using the SPSS package (SPSS 17.0 for Windows). The mean differences were compared using a least significant difference (LSD) test. Differences were considered significant when $P < 0.05$ (probability level). Each treatment was repeated three times with three plantlets per replication. The experiment was conducted in triplicate.

3. Results and discussion

3.1. Defense enzymatic activities

In the present investigation, patchouli was inoculated with the phytopathogenic bacterium *R. solanacearum*. Defense-related enzymes, namely POD, SOD, PPO and PAL, were extracted from patchouli leaves and stems at regular intervals of 24 h for 144 h, and the activities were determined. Results of POD, SOD, PPO and PAL are shown in Fig. 1, Fig. 2, Fig. 3, and Fig. 4, respectively. It was observed that in the control treatment (inoculated with sterile distilled water), the plant did not show any significant defense activity.

3.1.1. POD activity

In the infected upper leaves (Fig. 1a), a high level of POD activity was induced at 72 h.p.i. and was then maintained at higher levels than those of the corresponding controls during the experimental course. In the infected upper stems, the POD activity presented marked enhancement from 24 h.p.i. onward, before peaking at 72 h.p.i. (Fig. 1b). After reaching their highest level, the specific enzymatic activity started to decrease and thereafter returned to a relatively lower level at 120 and 144 h.p.i. In the infected lower leaves, the specific activity of POD did not achieve a noticeable increase until 48 h.p.i. and remained higher than the corresponding controls throughout the investigation period (Fig. 1c). In the infected lower stems (Fig. 1d), *R. solanacearum* inoculation elicited a biphasic POD accumulation with a low-amplitude transient first phase (24 h.p.i.), followed by a sustained phase of much higher magnitude that correlated with disease resistance, and exhibited a transient decrease at 120 h.p.i. before being restored to higher activity.

3.1.2. SOD activity

As shown in Fig. 2, the total SOD activity of infected patchouli increased within the first 24 h of exposure following *R. solanacearum* inoculation (*vs.* the un-inoculated control plants). The SOD activity of the infected plants remained relatively higher than those of the controls following inoculation before 120 h.p.i. In the infected upper leaves (Fig. 2a), there were three peaks with two transient decreases, while in the infected upper stems (Fig. 2b), two peaks with a temporary decrease were observed. In the infected lower stems and leaves, the SOD activity peaked at 24 h.p.i. and then gradually declined after that but was stably higher than that of the controls before 120 h.p.i. However, with prolonged pathogenic stress, SOD levels in the lower leaves and stems decreased compared with those in earlier phases or controls (Fig. 2a and d).

3.1.3. PPO activity

Fig. 3a and b indicates that PPO activity in infected patchouli leaves declined at 24 h.p.i. compared to their control counterparts. In the infected upper leaves (Fig. 3a), the PPO activity was activated in a

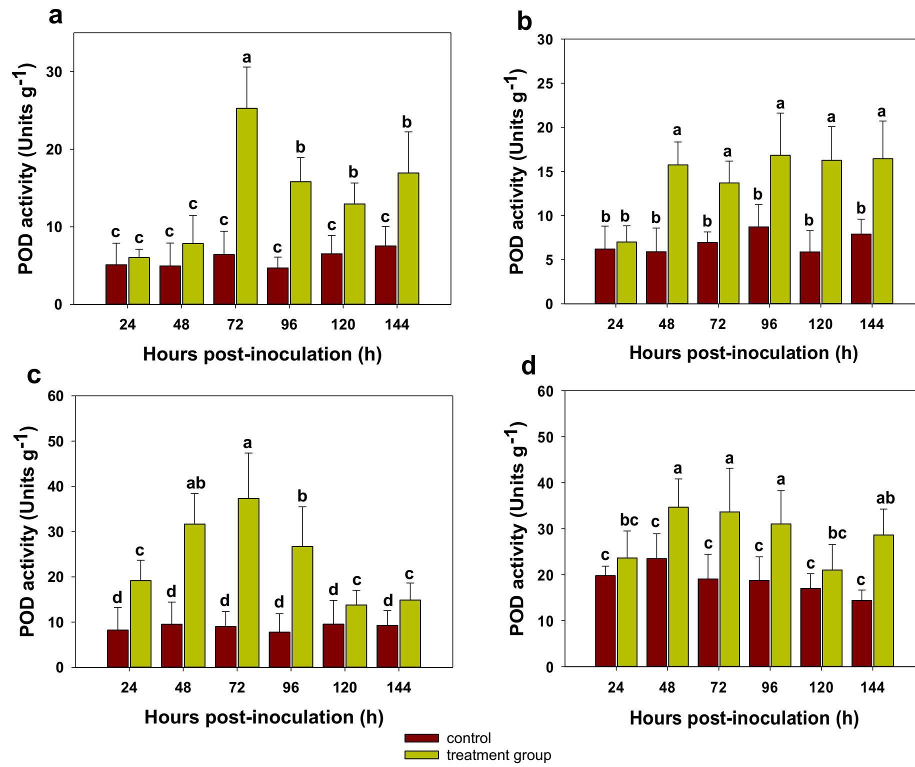

Fig. 1. Changes in peroxidase (POD) activity in the leaves and stems of patchouli inoculated with *R. solanacearum*. (a) Upper leaves; (b) upper stems; (c) lower leaves; (d) lower stems. Data in columns designated by the same letter(s) are not significantly different according to least significant difference test at $P = 0.05$.

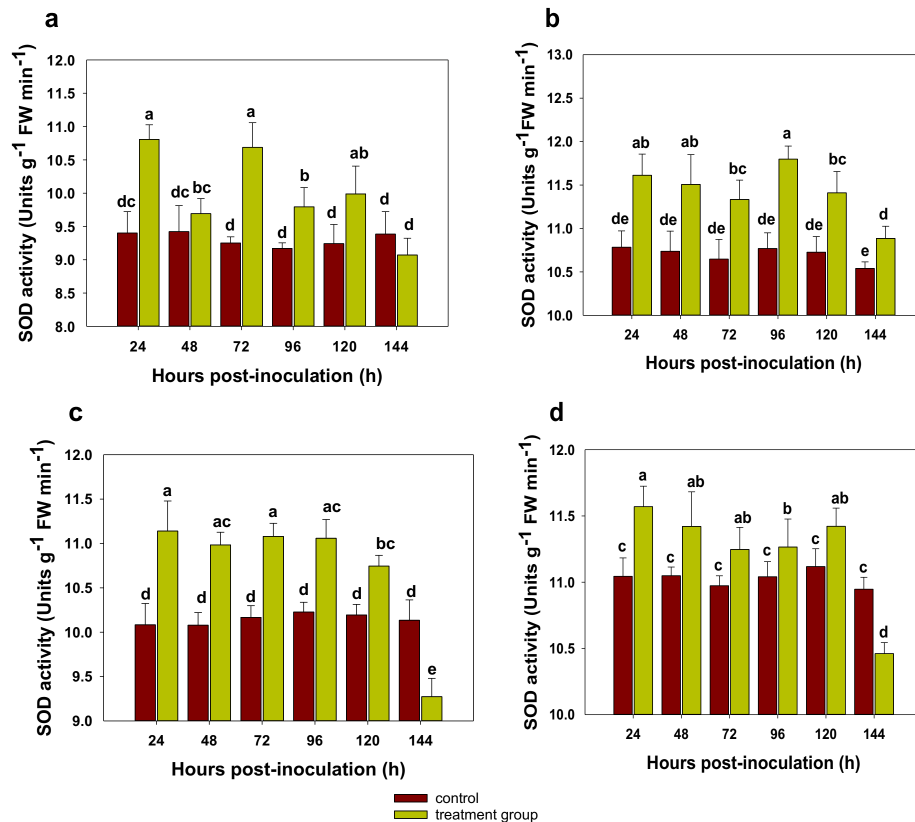

Fig. 2. Changes in superoxide dismutase (SOD) activity in the leaves and stems of patchouli inoculated with *R. solanacearum*. (a) Upper leaves; (b) upper stems; (c) lower leaves; (d) lower stems. Data in columns designated by the same letter(s) are not significantly different according to least significant difference test at $P = 0.05$.

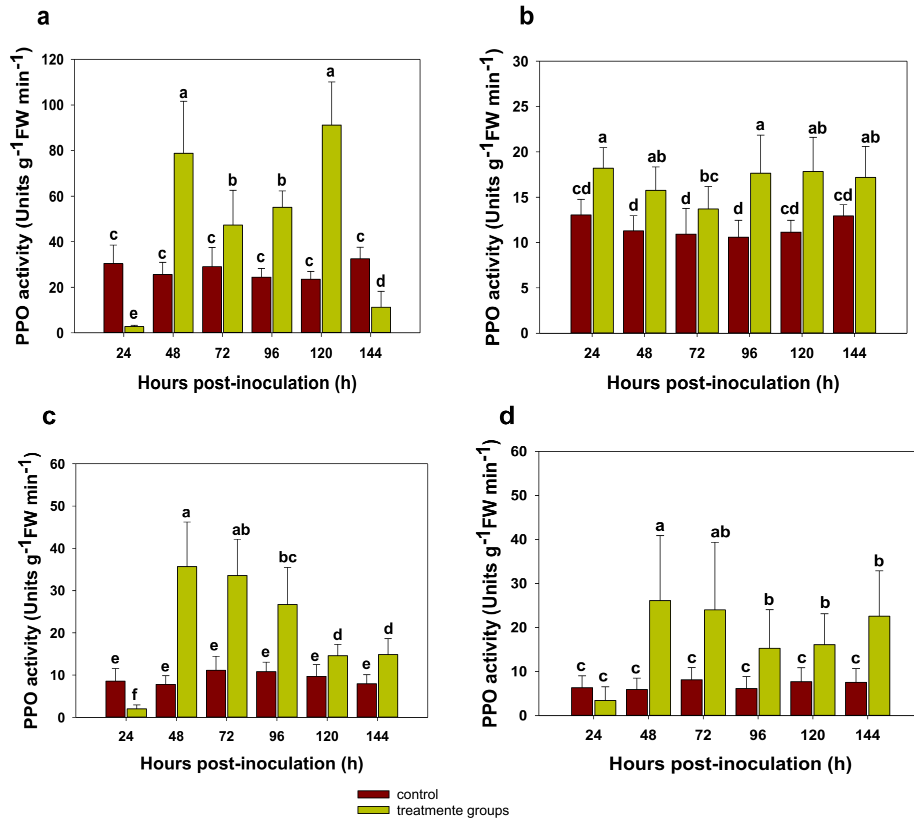

Fig. 3. Changes in polyphenol oxidases (PPO) activity in the leaves and stems of patchouli inoculated with *R. solanacearum*. (a) Upper leaves; (b) upper stems; (c) lower leaves; (d) lower stems. Data in columns designated by the same letter(s) are not significantly different according to least significant difference test at $P = 0.05$.

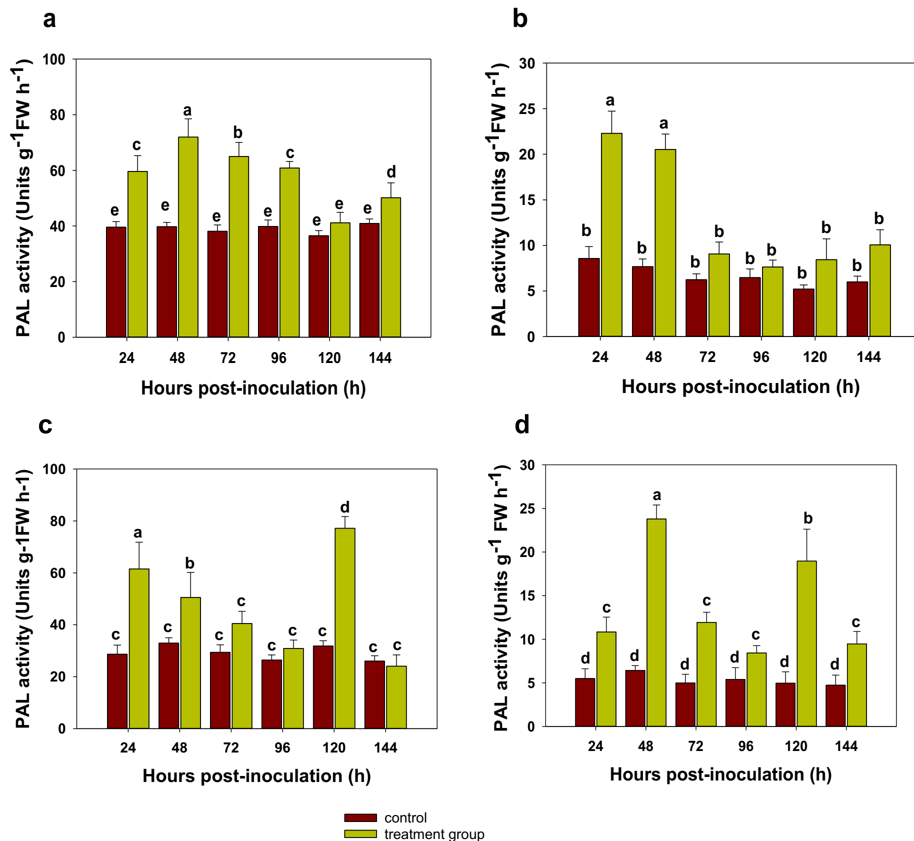

Fig. 4. Changes in phenylalanine ammonia-lyase (PAL) activity in the leaves and stems of patchouli inoculated with *R. solanacearum*. (a) Upper leaves; (b) upper stems; (c) lower leaves; (d) lower stems. Data in columns designated by the same letter(s) are not significantly different according to least significant difference test at $P = 0.05$.

biphasic manner, peaking at 48 and 120 h.p.i., with a transient decline at 72 and 96 h.p.i. At 24 and 144 h.p.i., the PPO activity decreased to a level lower than those in the corresponding controls. In the infected upper stems (Fig. 3b), PPO exhibited relatively less variation than other parts. However, PPO activities were stably higher than those of the corresponding controls. In the infected lower leaves (Fig. 3c), the specific activity of PPO was maintained at high levels, especially at 48, 72, and 96 h.p.i. In the infected lower stems also (Fig. 3d), the PPO activity was suppressed at 24 h.p.i but increased at 48, 72, and 144 h.p.i.

3.1.4. PAL activity

Fig. 4 illustrates that the PAL activity in leaves exhibited a greater increment than that in stems as a whole. In the infected upper parts (Fig. 4a and b), the PAL activity peaked at 48 h.p.i. and then declined. In the infected lower leaves (Fig. 4c), the PAL activity was high at 24 and 48 h.p.i. and then markedly declined between 72 and 96 h.p.i., before peaking again at a greater level at 120 h.p.i. In the infected lower stems (Fig. 4d), two high peaks appeared at 48 and 120 h.p.i. Then, the specific enzymatic activity started to decrease and thereafter returned to a relatively lower level at 72, 96, and 144 h.p.i. The PAL activity in the controls did not change significantly over the experimental course.

Regardless of the bacterial treatments, the lower leaves/stems showed higher POD and SOD activities but lower constitutive levels of PPO and PAL than their upper counterparts. It was also observed that PPO and PAL activities were significantly higher ($P < 0.05$) in leaves than in stems. Our findings were in accordance with a previous report using potato [23]. POD and SOD enzymatic activities significantly increased in patchouli inoculated with *R. solanacearum*, and the result was in line with the anti-oxidative isozyme analysis by Chai et al. [24] to some extent.

Distinctive bioprotective activity was also observed in the upper and lower leaves/stems of patchouli inoculated with *R. solanacearum*. This observation raised the possibility that patchouli could activate distinct protective responses tailored to specific compartments. This dynamic and differential response of defense enzymes might reflect the dynamic equilibrium of the defensive system within the plant, which corresponded to the stress of pathogen infection to some extent.

From the above observations, it was found that the overall defense-related enzymatic activities in patchouli were higher during the first 72 h of *R. solanacearum* inoculation. The enhanced defensive activities might contribute to increasing the resistance and alleviating the oxidative damage in patchouli against *R. solanacearum*. There were similar studies on the defense-related enzymatic response of plants against pathogens, such as on banana, rice, and grape [9,10,11]. However, with prolonged pathogenic stress, the overall level of arrays of defense-related enzymes was found to be lower.

3.2. Native gel electrophoresis analysis

Abundant POD and SOD isoforms exist in higher plants and play important protective roles against microbial infection. SOD and POD automatically constitute a mutually supportive team of defense against ROS [25] and operate in a well-balanced and coordinated manner in various cell compartments, accounting for disease resistance. To better understand the importance of these two enzymes, a native PAGE analysis of POD and SOD isoformic patterns was performed (Fig. 5 and Fig. 6).

The electrophoretic bands of POD are shown in Fig. 5. In inoculated plants, analysis of the isoenzymatic pattern of POD at 24, 48, and 72 h.p.i. displayed at least three isoformic bands, whose banding intensity was superior to that of the corresponding control. POD band 2, a novel and prominent band with intermediate electrophoretic mobility, was noticeably elicited by pathogen inoculation at 24, 48, and 72 h.p.i. and disappeared afterward. POD isoenzymes at 24, 48, and 72 h.p.i. were characterized by enhanced staining intensity,

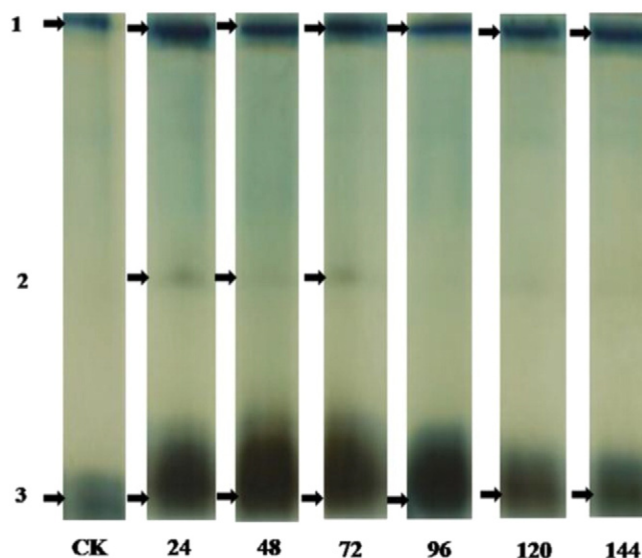

Fig. 5. Isoenzymatic bands of peroxidase (POD) from patchouli inoculated with *R. solanacearum*. Arrows depict all detected POD isoenzymes.

particularly bands 1 and 3, which corroborated with the enzymatic activity.

Fig. 6 indicates that there were two new SOD bands, namely SOD 1 and 3, identified distinctly at 24 and 48 h.p.i., and SOD 2 and 4 were intensified at 24, 48, and 72 h.p.i. SOD 1 with lower electrophoretic mobility and SOD 3 with higher electrophoretic mobility in the upper part of the gels were the distinctive isozymes to discriminate treatments and control. SOD 2 was intensified and predominantly present in infected plants and with intermediate mobility. SOD 4, with higher electrophoretic mobility, was also intensified after inoculation. Other bands were too weak to visualize.

As a whole, *R. solanacearum* inoculation enhanced the intensity of the existing isoforms and caused the synthesis of *de novo* isoforms of POD and SOD in patchouli. These results implied that not only the total activity of the enzymes but also their isoenzymatic patterns might exert an important effect in pathogen stress conditions.

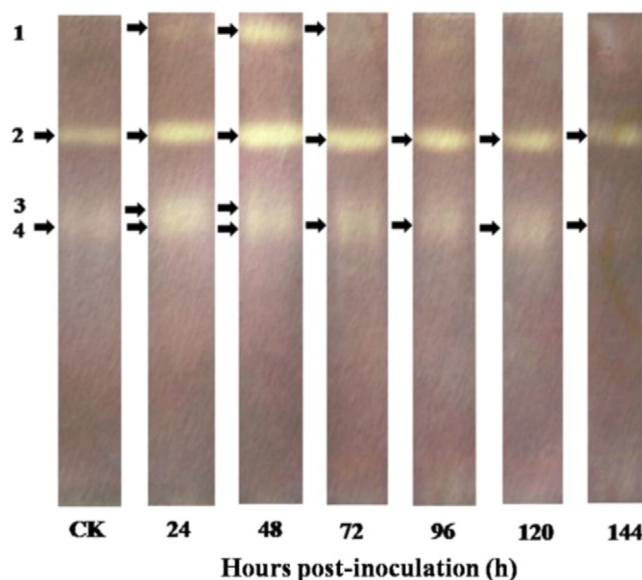

Fig. 6. Isoenzymatic bands of superoxide dismutase (SOD) from patchouli inoculated with *R. solanacearum*. Arrows denote all detected SOD isoenzymes.

4. Conclusion

On the basis of the present investigation, we conclude that the induced defense-related enzymes POD, PPO, PAL, and SOD of patchouli might function together as a protective mechanism to shield the plant from *R. solanacearum*.

Conflict of interest

Authors declare there is no conflict of interest.

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